09/622,353

WEST

| Help | Logout | Interrupt |

| Main Menu | Search Form | Posting Counts | Show S Numbers | Edit S Numbers | Preferences |

Search Results -

Term	Documents
PCR.DWPI,EPAB,JPAB,USPT.	24879
PCRS.DWPI,EPAB,JPAB,USPT.	681
(5 AND PCR).USPT,JPAB,EPAB,DWPI.	2

Database:

US Patents Full-Text Database
US Pre-Grant Publication Full-Text Database
JPO Abstracts Database
EPO Abstracts Database
Derwent World Patents Index
IBM Technical Disclosure Bulletins

| Refine Search: | 15 and PCR | Clear |

Search History

Today's Date: 6/17/2001

DB Name	Query	Hit Count	Set Name
USPT,JPAB,EPAB,DWPI	15 and PCR	2	L6
USPT,JPAB,EPAB,DWPI	14 and mutant	2	L5
USPT,JPAB,EPAB,DWPI	13 and maize	2	L4
USPT,JPAB,EPAB,DWPI	12 and transposable	2	L3
USPT,JPAB,EPAB,DWPI	Mutator and terminal inverted repeat sequence$1	2	L2
USPT,JPAB,EPAB,DWPI	Mutator near5 terminal inverted repeat sequence$1	0	L1

Term	Documents
PCR.DWPI,EPAB,JPAB,USPT.	24879
PCRS.DWPI,EPAB,JPAB,USPT.	681
(5 AND PCR).USPT,JPAB,EPAB,DWPI.	2

Display   10 Documents, starting with Document: 2

Display Format: CIT   Change Format

WEST

| Generate Collection |

Search Results - Record(s) 1 through 2 of 2 returned.

☐ 1.   Document ID: US 5981833 A

L6: Entry 1 of 2   File: USPT   Nov 9, 1999

US-PAT-NO: 5981833
DOCUMENT-IDENTIFIER: US 5981833 A

TITLE: Nuclear restorer genes for hybrid seed production

DATE-ISSUED: November 9, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Wise; Roger P.	Ames	IA	N/A	N/A
Schnable; Patrick S.	Ames	IA	N/A	N/A

US-CL-CURRENT: 800/271; 435/235.1, 435/412, 536/23.6, 536/24.1, 536/24.33, 800/275, 800/298, 800/303

| Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image |

☐ 2.   Document ID: US 5962764 A

L6: Entry 2 of 2   File: USPT   Oct 5, 1999

US-PAT-NO: 5962764
DOCUMENT-IDENTIFIER: US 5962764 A

TITLE: Functional characterization of genes

DATE-ISSUED: October 5, 1999

INVENTOR-INFORMATION:

NAME	CITY	STATE	ZIP CODE	COUNTRY
Briggs; Steven P.	Des Moines	IA	N/A	N/A
Meeley; Robert B.	Des Moines	IA	N/A	N/A

US-CL-CURRENT: 800/270; 536/23.6, 536/24.1, 536/24.33, 800/267, 800/275, 800/298, 800/320.1

| Full | Title | Citation | Front | Review | Classification | Date | Reference | Claims | KWIC | Draw Desc | Image |

| Generate Collection |

WEST

☐ | Generate Collection |

L6: Entry 1 of 2   File: USPT   Nov 9, 1999

DOCUMENT-IDENTIFIER: US 5981833 A
TITLE: Nuclear restorer genes for hybrid seed production

BSPR:
In plants, the best first filial (F.sub.1) hybrids have a substantial yield
advantage over the best open-pollinated varieties or inbred lines. This yield
advantage of a hybrid over its parents is termed "heterosis." The observed degree
of heterosis varies among species; however, as a general rule, it is high among
cross-pollinated species, such as maize and sunflower, and typically lower among
self-pollinated species, such as soybean and wheat.

BSPR:
In this regard, from at least about the 1940's, hybrid varieties of maize (a/k/a
corn) largely supplanted open-pollinated varieties because startling improvements
in yield, along with other agronomic traits, were realized when hybrid varieties.
were used. Indeed, the manufacture and sale of hybrid seed are the basis of a
significant agricultural industry.

BSPR:
In maize, an intermediate course historically has been taken. Two maize parents
have been grown in isolation from other potential maize parents, i.e., sources of
pollen. One parent was detasseled (emasculated) and served as the "female"
parent, whereas the other parent was allowed to produce pollen and fertilize the
female parent by cross-pollination, thereby serving as a "male" parent. Which
maize parent was chosen to be the female and which maize parent was chosen to be
the male were frequently based on commercially significant reasons. For example,
it was preferred to use maize with ample seed production as a female parent and
maize with ample pollen production as a male parent. Hybrid seed was then
harvested from the female parent.

BSPR:
An inbred line of maize can be converted into a CMS line by crossing it (as male)
to a known cytoplasmic male-sterile line and then backcrossing it (as female) to
the original inbred line. Given that the CMS-converted line is male-sterile, it
must be maintained by crossing by the original inbred line (a "maintainer").
Hybrid seed is produced by growing the CMS-converted inbred line and a second
inbred line in isolation, without detasseling.

BSPR:
Cytoplasmic male-sterile maize can be restored to fertility in a succeeding
generation by a nuclear restorer gene. For example, if a CMS-converted line is
grown in isolation with a second inbred line carrying a nuclear restorer gene,
then the F.sub.1 will be male-fertile and potentially economically valuable.

BSPR:
There are three types of male-sterile cytoplasms in maize (Zea mays L.): S
(USDA), C (Charrua), and T (Texas). These three male-sterile cytoplasms can be
distinguished by the ability of different nuclear (restorer) genes to restore
fertility to the plants with these different cytoplasms (see Laughnan et al.
(1983), supra), by mitochondrial DNA restriction endonuclease profiles (see Pring
et al., Genetics, 89, 121-136 (1978)), and by .sup.35 S-methionine-labeled
polypeptides translated in isolated mitochondria (see Forde et al., PNAS USA, 75,
3841-3845 (1978)).

BSPR:
In contrast to the male-sterile cytoplasms, the normal (N), male-fertile

cytoplasm yields fertile plants in either the presence or absence of all known nuclear backgrounds, whereas the male-sterile C, S, and T cytoplasms only produce fertile plants in nuclear backgrounds carrying the appropriate restorer genes. These nuclearly encoded, fertility-restorer genes compensate for cytoplasmic dysfunction(s) that are phenotypically expressed during microsporogenesis and/or microgametogenesis. Plants carrying S or C cytoplasm are restored to fertility by a single dominant allele of the rf3 or rf4 locus, respectively. Preliminary evidence suggests that the rf3 locus is flanked by whp and bn117.14 on chromosome 2L (Kamps et al., Maize Genet. Coop. Newsl., 66, 45 (1992)). The rf4 locus maps to chromosome 8, approximately 2 cM from the RFLP ("restriction fragment length polymorphism") marker NP1114A (Sisco, Crop Sci., 31, 1263-1266 (1991)). In contrast to S and C cytoplasms, plants with T cytoplasm are restored to fertility by the dominant alleles of two loci, rf1 and rf2 (Laughnan et al. (1983), supra; and Levings et al., Plant Cell, 5, 1285-1290 (1983)), which are located on separate chromosomes. The rf1 locus is flanked by umc97 and umc92 on chromosome 3, and the rf2 locus is flanked by the umc153 and sus1 on chromosome 9 (Wise et al., Theor. Appl. Genet., 88, 785-795 (1994)).

BSPR:
CMS also occurs in other species of plants. Examples of other species of plants include petunia (Nivison et al., Plant Cell, 1, 1121-1130 (1989)), the common bean (Janska et al., Genetics, 135, 869-879 (1993)), Brassica napus (Singh et al., Plant Cell, 3, 1349-1362 (1991)), sunflower (Laver et al., The Plant Journal, 1, 185-193 (1991)), sorghum (Bailey-Serres et al., Theor. Appl. Genet., 73, 252-260 (1986)), and oats (Mann et al., Theor. Appl. Genet., 78, 293-297 (1989)). Like S-cytoplasmic maize, cytoplasmic male sterility in petunia, beans, and Brassica can be restored to fertility by single dominant nuclear genes.

BSPR:
In T-cytoplasmic maize, CMS is associated with the unique mitochondrial gene T-urf13 (Wise et al., PNAS USA, 84, 2858-286 (1987a)). Toxin sensitivity traits are also associated with this gene (Huang et al., EMBO, 9, 339-247 (1990)). T-urf13 encodes a 13 kDa mitochondrial polypeptide (URF13) (Wise et al., Plant Mol. Biol., 9, 121-126 (1987b)), which is located in the mitochondrial membrane (Dewey et al. (1987), supra) and appears to span the mitochondrial membrane in oligomeric form (Korth et al., PNAS USA, 88, 10865-10869 (1991)). URF13 is not synthesized by deletion mutants (Dixon et al., Theor. Appl. Genet., 63, 75-80 (1982)), is truncated in the T4 frameshift mutant (Wise et al. (1987b), supra), and binds to fungal pathotoxins (Braun et al., Plant Cell, 2, 153-161 (1990)).

BSPR:
T cytoplasm was used predominantly in the late 1960's because of its reliability. The other male-sterile cytoplasms of maize, namely C and S, tended to "break down" in the field, i.e., self-pollination or incomplete fertility restoration occurred. Thus, approximately 85% of the hybrid maize seed in the U.S. was T-cytoplasm until the epidemic of southern corn leaf blight, which occurred in 1970 (Pring et al., Ann. Rev. Phytopathol., 27, 483-502 (1989)).

BSPR:
After the 1970 epidemic, it was determined that T-cytoplasmic maize is highly sensitive to the host-selective toxin (T toxin) produced by race T of the fungus Cochliobolus heterostrophus Drechsler (asexual stage Bipolaris mayadis Nisikado and Miyake), which is the causal organism of southern corn leaf blight (Comstock et al., Phytopathology, 63, 1357-1361 (1973)). T-cytoplasmic maize was also found to be highly sensitive to the host-selective toxin (Pm toxin) produced by another fungus, namely Phyllosticta maydis, Arny and Nelson, which causes yellow leaf blight (Yoder, Phytopathology, 63, 1361-1366 (1973)).

BSPR:
A focus of research since the 1970's has been to develop alternative genetic approaches to emasculating plants for the purpose of hybrid seed production. This effort, in part, reflects a desire among farmers to maintain some level of genetic heterogeneity for any given crop. One approach (Marc Albertsen, Pioneer Hi-Bred International, Inc.) involves the use of nuclear male-sterile genes. This particular approach, which is predicated, at least in part, on earlier analogous work with Arabidopsis (see Aarts et al., Nature, 363, 715-717 (1993)), specifically uses a cloned nuclear male-sterile gene from maize, although there are a number of such genes in a given plant species, including maize (Albertson et al., Can. J. Genet. Cytol., 23, 195-208 (1981)). An inbred line of maize, for

example, which is homozygous for a <u>mutant</u> allele of a nuclear male-sterile (ms)
gene, is genetically engineered to carry a construct comprising an inducible
promotor, which, upon induction, allows expression of a wild-type ms gene. The
inbred line is maintained in isolation, where it is sprayed with the inducer and
allowed to self- and sib-pollinate. Hybrid seed is produced by growing the inbred
line with a second inbred line, which carries a wild-type allele of the ms gene,
in isolation and in the absence of inducer. Accordingly, the F.sub.1 is
heterozygous and, therefore, fertile. This approach is disadvantageous, however,
in that it requires maintenance of the male-sterile line and the use of an
inducer.

BSPR:
In another embodiment of a method of producing hybrid seed provided by the
present invention, a first plant, which is homozygous for a leaky <u>mutant</u> allele
of Rf1 or Rf2, and a second plant, which comprises a defective cytoplasm as
defined herein, are generated. Then, the second plant is pollinated by the first
plant, and a seed, which generates a fertile plant, is obtained from the second
plant. Alternatively, a seed is obtained from the second plant and is grown into
a sterile plant, the male-sterile progeny of which are then backcrossed by a
plant like the first plant until a plant, which is homozygous for the leaky
<u>mutant</u> allele of Rf1 or Rf2, is obtained. Accordingly, a variant of a cytoplasmic
male-sterile plant is provided.

DRPR:
FIG. 1 is a comparison of RNA processing sites between <u>maize,</u> sorghum, archaea
and rice.

DEPR:
The term "nucleic acid" refers to a polymer of DNA or RNA, i.e., a
polynucleotide, which can be single- or double-stranded, and can optionally
contain synthetic, nonnatural, or altered nucleotides. Any combination of such
nucleotides can be incorporated into DNA or RNA polymers. The nucleic acid is
"enriched" in that the concentration of the material is at least about 2, 5, 10,
100, or 1,000 times its natural concentration, for example, advantageously about
0.01% by weight, preferably at least about 0.1% by weight. Enriched preparations
of about 0.5%, 1%, 5%, 10%, and 20% or more by weight are also contemplated. A
nucleic acid is "isolated" in that the material has been removed from its
original environment, e.g., the genome of a plant, presuming that it is naturally
occurring. Thus, describing the nucleic acid of the present invention as
"substantially isolated" reflects the increase in concentration of the nucleic
acid of interest with respect to other nucleic acids, as when, for example, the
nucleic acid of interest is taken from a plant (which, in the case of <u>maize,</u> has
a complement of three million kb) and is placed or cloned into a bacteriophage
(which, in the case of lambda (".lambda.") has a complement of 50 kb), resulting
in a 60,000-fold increase in concentration of the nucleic acid of the present
invention with respect to the total amount of DNA in the bacteriophage into which
it is placed. It is also advantageous that the nucleic acids be in purified form,
or substantially purified form, wherein "purified" does not mean absolute purity
but, rather, relative purity, wherein, for example, the nucleic acids of the
present invention are isolated in a laboratory vessel in a mixture of other
nucleic acids, such as portions of a vector or other molecules associated with
genetic engineering.

DEPR:
Preferably, the enriched or substantially isolated nucleic acid hybridizes under
at least moderately stringent hybridization conditions to a second nucleic acid
comprising a nucleotide sequence specific to a plant-derived restorer gene (Rf),
or a substantial portion thereof; more preferably, the nucleic acid hybridizes
under the aforementioned conditions to a second nucleic acid comprising a
nucleotide sequence specific to the Rf1 or Rf2 gene of <u>maize,</u> or a substantial
portion thereof.

DEPR:
The nucleic acid of the present invention can be isolated from any suitable,
sexually-reproducing plant, which can be monocotyledonous or dicotyledonous, as
long as the plant comprises an Rf gene as described herein. Preferred plants
include <u>maize,</u> wheat, barley, rice, common bean, oats, rye, soybean, rapeseed,
canola, cotton, safflower, peanut, palm, sorghum, sunflower, beet, tomato,
cucumber, petunia and other ornamental flowers. The more preferred plant from

which the Rf gene is derived is <u>maize</u>.

DEPR:
SEQ ID NO: 1 is a partial sequence (402 bases sequenced out of a total of about 1200 bases;) of the cDNA derived from the <u>maize</u> Rf2 gene, the identification and isolation of which are described in Examples 1 and 2. SEQ ID NO: 1 does not appear to be a full-length cDNA (i.e., including the complete complement of the Rf2 mRNA) because, as compared to the evidently homologous aldehyde dehydrogenase gene sequences disclosed in Example 4, approximately 500 bp of the Rf2 message is missing. Nevertheless, one of ordinary skill in the art certainly recognizes that either one of SEQ ID NO: 1 and SEQ ID NO: 3 provides sufficient Rf2 sequence information to provide probes for the identification and cloning of any Rf2 gene or to any gene that is substantially homologous to Rf2 in sequence, such that the Rf2 probe hybridizes under moderately stringent to stringent hybridization conditions, as discussed herein above. Accordingly, the present invention also provides Rf2-specific probes.

DEPR:
SEQ ID NO: 4 is a sequence of a total of about 2473 bases, including poly A, of the cDNA derived from the <u>maize</u> Rf1 gene, the identification and isolation of which are described in Examples 6-8. Accordingly, the present invention also provides Rf1-specific probes.

DEPR:
A nucleic acid can be identified for enrichment or substantial isolation by hybridization to any subfragment of SEQ ID NOS: 1, 3 or 4 of at least 20 nucleotides under stringent hybridization conditions as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (2d ed., 1989). Accordingly, this invention encompasses the entire sequence of the Rf1 and Rf2 genes and fragments thereof, which have been generated by any suitable technique, such as by restriction enzyme digestion of chromosomal or plasmid DNA, by <u>PCR,</u> or by synthesis, and which can be either DNA or RNA.

DEPR:
Chromosome walking is a particularly useful technology that can facilitate the molecular isolation of any mapped gene (Bender et al., J. Mol. Biol., 168, 17-338 (1983)) and has been found to be particularly useful with plants that have a relatively small genoine size, such as that of Arabidopsis. This technique is useful, of course, in species of larger genome size as well, such as <u>maize</u>.

DEPR:
A chromosome walk is initiated by identifying from a library of large DNA fragments the specific fragment(s) that contain sequences homologous to a restriction fragment length polymorphism (RFLP) marker or some other marker that is closely linked to the target gene. Typically, the library of DNA fragments is maintained as yeast artificial chromosomes, i.e., YACs (Burke et al., Science, 236, 806-811 (1987)), although cosmids, P1 phage or .lambda. phage have been used. Single copy sequences from the termini of YACs that contain sequences homologous to a starting RFLP are then used as hybridization probes to isolate overlapping DNA fragments. This process is repeated until the entire chromosomal region, from the starting RFLP marker to beyond the target gene, has been cloned as a contiguous segment (a "contig"). Typically, the contig is oriented by mapping DNA sequences from the growing contig to the genetic/RFLP map. Similarly, the endpoint of the walk is established by demonstrating that the contig contains DNA sequences from both sides of the target gene. For both of these operations, DNA sequences from the contig must be genetically mapped. The efficiency of this mapping can be greatly increased by selecting a population of plants that have a high probability of carrying recombination breakpoints in the region defined by the contig. Such a mapping population is established by selecting plants that carry a recombination breakpoint between two visible genetic markers that flank the interval to be walked. The precision of the mapping increases proportionally with the number of genetic recombinants. The greater the precision of this mapping, the smaller the uncertainty associated with the positioning of the target gene on the contig. Once the target gene has been localized in the contig to as small an interval as the mapping population permits, the target gene is identified from the interval via its ability to complement genetically the <u>mutant</u> phenotype. The ability of a sequence to complement the <u>mutant</u> phenotype is assayed by transforming plants homozygous for a <u>mutant</u> allele of the target gene. Alternatively, comparisons between wild-type And <u>mutant</u> sequences also can

identify the target gene from the interval.

DEPR:
Other technologies for gene isolation in Arabidopsis and other plants include
genomic subtraction, and transposon and T-DNA tagging. Genomic subtraction
requires the availability of strains having deletions of the target gene (Strauss
and Ausubel, PNAS USP, 87, 1889-1893 (1990); and Sun et al., Plant Cell, 4,
119-128 (1992)); however, such deletions are not available for rf2, for example.
A transposon tagging system in Arabidopsis has recently become available. The
success in tagging and cloning a petunia gene using a heterologous maize
transposon (Chuck et al., Plant Cell, 5, 371-378 (1993)) provided the direction
to extend this technique to Arabidopsis, and further demonstrates that this
technique is amenable to tagging virtually any plant with heterologous (Dean et
al., Plant J., 2, 69-81 (1992); Grevelding et al., PNAS USA, 89, 6085-6089
(1992); Swinburne et al., Plant Cell, 4, 583-595 (1992); and Fedoroff and Smith,
Plant J., 3, 273-289 (1993)) and/or endogenous (Tsay et al., Science, 260,
342-344 (1993)) transposons. T-DNA tagging has been realized (Feldmann, Plant J.,
1, 71-82 (1991)) and is in wide use (e.g., Feldmann et al., Science, 243,
1351-1354 (1989); Herman et al., Plant Cell, 11, 1051-1055 (1989); Konz et al.,
EMBO J., 9, 1337-1346 (1989); and Kieber et al., Cell, 72, 427-441 (1993)).
Additionally, having isolated at least two nuclear restorer genes, the nucleic
acid thereof can be used whole or in parts (by sub-cloning fragments thereof) as
a probe in heterologous systems. Preferably, such a technique requires that the
stringency of the selective hybridization procedure be lowered, and then slowly
raised, as is well known in the art.

DEPR:
Although T-DNA tagging, chromosome walking or heterologous probe selection can
identify a DNA fragment that putatively contains the gene of interest, in each
instance these DNA fragments must be confirmed by genetic complementation or some
other means, which is fully disclosed in the examples. Although the methods of
identification of a particular gene sequence have been described largely herein
with reference to maize and Arabidopsis only, it is abundantly clear to one of
ordinary skill that such methods can be adapted for gene identification in other
species, particularly in the context of the present invention. Accordingly, the
identification of the Rf genes, and cloning and use thereof, is enabled hereby
for any of the aforementioned sexual-reproducing plants, as well as other plants
that have mitochondrial deficiency-derived phenotypes in need of amelioration or
correction, in particular those mitochondrial deficiencies amenable to
amelioration or correction by one or more of the nucleic acids described herein.

DEPR:
The method of detection involves duplex formation by annealing or hybridization
of the oligonucleotide probe, either labeled or unlabeled, depending upon the
nature of the detection system, with the DNA or RNA of an organism believed to
produce the particular nuclear restorer gene. Usually this method of detection
involves cell lysis, extraction of nucleic acids with organic solvents,
precipitation of nucleic acids in an appropriately buffered medium, and isolation
of the DNA or RNA. Alternatively, one can amplify specific sequences via
polymerase chain reaction (PCR). The DNA can be fragmented by mechanical shearing
or restriction endonuclease digestion. The nucleic acid can then be bound to a
support or can be used in solution, depending upon the nature of the protocol.
The Southern technique (Southern, J. Mol. Biol., 98, 503 (1975)) can be employed
with denatured DNA by binding the single-stranded fragments, for example, to a
nitrocellulose or nylon filter. RNA also can be blotted onto a filter (Thomas,
PNAS USA, 77, 5201 (1980)). Preferably, the fragments are subjected to
electrophoresis prior to binding to a support so as to enable the selection of
variously sized fractions. Alternatively, the assay can be accomplished on plant
cells fixed to a substrate and permeabilized by methods known in the art,
whereupon the hybridization procedure can be conducted to determine if a
homologous gene to a particular nuclear restorer gene exists in the plant of
interest, and/or if that plant is expressing RNA that is homologous to the
nuclear restorer gene.

DEPR:
The method is applicable to any plant, preferably any sexually reproducing plant,
including plants of agronomic value, such as maize, soybean, alfalfa, wheat,
rapeseed, rice, sorghum, beet, various vegetables including cucumber, tomato,
peppers, and the like, various trees including apple, pear, peach, cherry,

redwood, pine, oak, and the like, and various ornamentals. The plant is
preferably <u>maize,</u> rapeseed, rice or sorghum.

DEPR:
Hybrid seed also can be produced in accordance with another aspect of the present
invention. For purposes of setting forth the general method, Rf2 will be used as
an example; the method, of course, is not limited to Rf2. Using methods generally
known in the art, e.g., backcrossing, mutagenesis, or homologous recombination, a
first plant, which is homozygous for a leaky <u>mutant</u> allele of Rf2 (or another
housekeeping gene product present in the mitochondria, the chloroplast or
cytosol), is generated. The leaky <u>mutant</u> allele can, for example, comprise minor
amino acid substitutions that only slightly impair the function of the
housekeeping gene product. Alternatively, the leaky <u>mutants</u> can arise via altered
regulatory sequences, such that the gene product is present at somewhat less than
optimal amounts. Such an allele should condition male fertility in a normal
cytoplasm but cause or allow male sterility in certain novel cytoplasms. In
addition, the leaky allele preferably hybridizes to a nucleic acid described
above under at least moderately stringent conditions. A second plant, which
comprises a "defective" cytoplasm due to one or more genetic defects, which
condition male fertility in a completely wild-type nuclear genome, but male
sterility in the presence of a nuclear genome homozygous for a leaky <u>mutant</u> Rf
allele, is also generated, using such methods as described with respect to the
first plant. The second plant is then crossed by, i.e., pollinated by, the first
plant to obtain seeds, which generate fertile and sterile F.sub.1 plants, from
the second plant. F.sub.1 seeds that produce sterile plants can be grown and
backcrossed by plants like the above-described first plant to homozygosity for
the leaky <u>mutant</u> alleles of Rf1 or Rf2. Plants with the defective cytoplasm and
homozygous for the leaky allele will be male-sterile and, therefore, useful for
the production of hybrid seed, as set forth herein above. Taking into
consideration the possible detoxifying function of the Rf2 gene product, the same
procedures can be followed wherein the housekeeping gene function is to restore
the activity of a second gene product's activity by, for example, removing
aldehyde groups that are destabilizing or otherwise improve the functioning of
the second gene product.

DEPR:
This example illustrates the use of two different transposon systems in <u>maize</u> for
the selection of lines of plants that carry male-fertility nuclear restorer
genes.

DEPR:
The following populations of <u>maize</u> were used to map the chromosomal locations of
the rf1 and rf2 genes. For further discussion of the sources of the populations
of <u>maize</u> and the genetic crosses performed, see Wise et al., Theor. Appl. Genet.,
88, 785-795 (1994).

DEPR:
The RFLP maps of <u>maize</u> chromosomes 3 and 9, along with other such maps, were used
in crosses between a transposon-carrying line and a genetically marked
nontransposon line to determine the position of a transposon on a given
chromosome by insertional mutagenesis as evidenced by analysis of RFLP and
visible markers. Accordingly, stocks carrying certain transposon systems, i.e.,
<u>Mutator,</u> Cy, or Spm, were crossed to genetically marked nontransposon lines in
order to tag the Rf1 and Rf2 genes (see Walbot, Ann. Rev. Plant Physiol. Plant
Mol. Biol., 43, 49-82 (1992)). Specifically, Rf2 was tagged in the following
cross, which was carried out in an isolation plot using the method of Peterson
(In <u>Maize</u> Breeding and Genetics (D. B. Walden, ed., John Wiley & Sons, New York,
N.Y., 1978), pages 601-631) (the female parent is listed first in all crosses
herein):

DEPR:
Accordingly, transposon-generated <u>mutants</u> at the Rf2 genetic locus were obtained
for use in isolating the Rf2 gene <u>and/or</u> cDNA.

DEPR:
Using standard recombinant DNA techniques, total DNA from a single male-sterile
plant that carried rf2-m8122 was subjected to preparative Eco RI/Hin dIII
digestion and size-selected DNA was isolated for ligation into the lambda phage
vector NM 1149 (Murray, The Bacteriophage Lambda, Hendrix, ed. (1983)). The 3.4

kilobase Eco RI/Hin dIII fragment released from a Mu1-positive lambda phage
(named .lambda.91 8122 #9) isolated from the resulting library was subcloned into
the plasmid vector pBSK or pBKA (Stratagene), and named prf2. The prf2 plasmid
was used to transform E. coli DH5.alpha. or XL1-Blue MRF', and named PF#9. A
restriction site map of the insert of the pRf2 plasmid is shown in FIG. 2,
wherein H stand s for Hin dIII, D stands for Dra I, Hc stands for Hin cII, P
stands for Pst I, Bg stands for egt II, and E stands for Eco RI. The location of
the Mu1 insertion point is clearly marked as between the second Dra I and Bgl II
restriction sites. Hybridization of the radiolabeled pRf2 fragment #1 to maize
genomic DNA established that the cloned DNA was derived from the 3.4 kb
Mu1-hybridizing Eco RI-Hin dIII fragment present in male-sterile but not
male-fertile siblings.

DEPR:
The identity of the Rf2 genomic clone described above was confirmed by allelic
cross-referencing experiments. A single-copy fragment from pRf2 (i.e., rf2 probe
#1, FIG. 2) was hybridized to DNA derived from five independent rf2 mutants and
their respective wild-type progenitor alleles as recited in Example 1. The
results revealed polymorphisms between each rf2-m allele and its corresponding
wild-type progenitor allele. Specifically, the rf2-m8122 allele was associated
with a slower-moving restriction fragment (i.e., a fragment with lower gel
electrophoretic mobility) than was the progenitor Q66 allele. Similarly,
rf2-m8110 differed from its progenitor Q67, rf2-m9323 differed from its
progenitor Q66, and rf2-m9390 differed from its progenitor Q67. The results
confirmed that pRf2 included at least a portion of the sequence of the Rf2 gene.

DEPR:
Using .sup.32 P-radiolabeled rf2 probes #1 and #2 (shown in FIG. 2) in
combination, a cDNA library derived from mRNA isolated from immature maize
tassels and cloned into .lambda.GT10 phage was screened, using methods well-known
in the art (see Sambrook et al. (1989), supra; the cDNA library, named ts2, was a
gift of S. Delaporta of Yale University). From a first screen, six putative
positive plaques of phage were identified, which, upon a series of two
rescreenings, were resolved to include three verified positive plaques. DNA from
the positive phage was analyzed for its ability to hybridize to probes #1 and #2.
In addition, the sizes of the inserts were determined. The phage having the
largest insert was selected for subcloning into plasmids pBSK and pBKA
(Stratagene) for sequencing. The cDNA clone used for sequencing was named rf2
cDNA 6-2-8-1.

DEPR:
The present invention can be used to generate multiple CMS systems, which can be
mixed in the same manner as the C, S and T systems are now, thereby providing a
greater variety of genetic backgrounds for hybrid seed production. Because Rf2
appears to encode a common enzymatic function, aldehyde dehydrogenase, "leaky"
mutations (such as that presumably carried by the rf2-ref allele) are not
phenotypically expressed in a normal cytoplasm. For example, an N-cytoplasmric
rf2/rf2 plant is male-fertile. However, in a male-sterile cytoplasm, such as T,
which is already "weakened," the rf2 mutation and the cytoplasmic mutation, in
combination, effectively result in male sterility. Accordingly, an existing
male-sterile cytoplasm, such as C, S or T, or a mutated cytoplasm and a mutant or
mutated gene, like rf2, which is associated with a mitochondrial or cytosolic
function, are combined. In combination, these two mutations serve as a
CMS/restorer system. Any number of CMS/restorer systems can be made and used in
any species. This broad cross-species applicability of the present invention is
evident in view of the degree of homology between SEQ ID NO: 1 of maize and
nucleic acid sequences encoding proteins in such an evolutionarily divergent
organism as the cow.

DEPR:
In addition, "strong" mutants (e.g., complete loss of function) of rf2 can be
generated so as to render a normal cytoplasm phenotypically male-sterile (upon
self-pollination). An example of such an allele is one of the transposon-induced
alleles described in Schnable et al., Genetics, 136, 1171-1185 (1994). Such
alleles can be used, for example, in the Pioneer system described above.

DEPR:
Rf1 clones were isolated as described for the isolation of Rf2 clones, i.e., a
transposon-carrying line was used to tag the Rf1 gene. It is necessary to use

linked markers (as identified in the map of <u>Maize</u> Chromosome 3 above) to
distinguish between the newly induced <u>mutants</u> and the recessive allele used to
uncover them. To facilitate this approach, five rf1 inbreds (W22, B37, Mo17, W64A
and B73) were RFLP-fingerprinted for chromosome 3. Their respective DNAs were
digested with 8 restriction endonucleases (Bam HI, Eco RI, Eco RV, Hin dIII, Kpn
I, Dra I, Bcl I, and Bgl II), followed by Southern analyses with RFLP markers
that flank rf1, in order to identify an rf1 donor line with distinctive RFLP
alleles flanking rf1. By so doing, it was established that rf1-B37 can easily be
distinguished from the Rf1Ky21 or Rf1-IA153 (present in the Wf9-BG inbred)
alleles present in the <u>Mutator</u> population by using the restriction enzyme Dra I
in conjunction with umc10 and umc92, which flank rf1 (see above).

DEPR:
Using the above strategy and materials, <u>Mutator</u>-induced male-sterile <u>mutants</u> of
the rf1 fertility-restorer locus were identified. The Rf1/Rf1 <u>Mutator</u> lines were
crossed by the inbred line B37, which has the genotype rf1/rf1, Rf2/Rf2, thereby
providing Cross 4:

DEPR:
In the absence of mutation, the progeny from cross 4 are male-fertile because,
although they have T cytoplasm, they carry at least one copy of each of the two
dominant nuclear restorer factors, Rf1 and Rf2. However, if, with respect to a
given progeny, the Rf1 locus was inactivated by insertion of a Mu element, then
that plant would be male-sterile. These exceptional plants putatively carry
<u>Mutator</u>-induced rf1-m alleles. Progeny of Cross 4 (.about.=123,500) were screened
for mutations at rf1; ten putative male-sterile <u>mutants</u> were identified (Wise et
al., Genetics, 143, 1383-1394 (July 1996)).

DEPR:
To confirm if the putative male-sterile <u>mutants</u> were heritable and to determine
if the male-sterile phenotype was associated with the rf1 locus, the putative
male-sterile <u>mutants</u> from Cross 4 (with the predicted genotype of T cytoplasm,
rf1-m/rf1-B37, Rf2/Rf2) were crossed as shown in Cross 5:

DEPR:
T rf1-m rg1+/rf1-B37 rg1+.times.T Rf1 Rg1/rf1 rg1+, wherein rg1+ refers to the
normal wild type allele found in most <u>maize</u> lines. Plants with the genotype rf1-M
rg1+/Rf1 Rg1 were predicted to constitute 25% of the progeny from Cross 5 and
were identified by the ragged phenotype and previously characterized DNA
polymorphisms at chromosome 3 of each of Wf9-BG, Ky21, B37 and the previously
described Rf1 Rg1 stock. Based on their pedigree, rf1-m alleles were expected to
couple with Ky21- and/or Wf9-BG-derived RFLP markers, which are easily
distinguished from those flanking the rf1 allele in B37. Plants derived from
Cross 5 and having this target genotype were crossed as males onto T-cytoplasmic
W64A females (rf1/rf1 Rf2/Rf2, see cross 6) as follows: Cross 6: T rf1/rf1
(W64A).times.T rf1-m rg1+/Rf1 Rg1. Heritable, male-sterile mutations from Cross 6
were expected to segregate 1:1 (male-sterile : male-fertile), whereas
non-heritable, male-sterile "mutants" were expected to be male-fertile. If the
male-sterile mutation were at the rf1 locus, male-sterile plants would be normal
and male-fertile plants would be ragged (except for rare crossovers). Four of the
ten putative male-sterile <u>mutants</u> described above were shown to represent rf1-m
alleles.

DEPR:
The families resulting from Cross 6 and carrying rf1-m3207 and rf1-m3310
segregated 1:1 for male-sterile, normal plants (rf1-m rg1+/rf1-B37 rg1+) and
male-fertile, ragged (Rf1 Rg1/rf1-B37 rg1+) plants. DNA from each individual
sibling was digested for cosegregation analysis with Hin dIII and/or Eco RI.
Southern blots of the digested DNA were then probed with radiolabeled
Mu1-specific sequences. For each of these rf1-m alleles, a Mu1-containing DNA
fragment was identified that cosegregated with the <u>mutant</u> allele in over 40
progeny and, therefore, had a higher probability of representing Rf1 sequences.
These DNA fragments were then cloned using procedures described in Example 2, and
related procedures well-known in the art. Allelic cross-referencing experiments,
as described above in Example 3, were then used to establish that the cloned
sequences, indeed, represented Rf1.

DEPR:
In DNA gel blot cosegregation analyses, the rf1-m3207 and rf1-m3310 alleles

cosegregated with 5.5- and 2.4-kb Mul-hybridizing Eco RI restrictions fragments, respectively, suggesting that they contained Mu transposon insertions in the Rfl gene (Wise et al. (1996), supra). Following these analyses, total DNA from single male-sterile plants carrying the rfl-m3207 and rfl-m3310 alleles were subjected to preparative Eco RI digestion and size-selected DNA was isolated for ligation into the Eco RI site of Lambda ZapII (Stratagene, La Jolla, Calif.). Ligations were packaged with Gigapack III Gold packaging extracts (titres ranged from 2.times.10.sup.8 to 1.times.10.sup.9 pfu/.mu.g) and plated onto NZCYM agar in 24.times.24 cm sterile Petri plates (Nunc) with Mg-top agarose and incubated at 37.degree. C. until the plaques were visible (7-8 hr). Plaques were lifted onto Hybond N+ and hybridized with a random-primed Mul probe. Mul-hybridizing plaques were purified and Bluescript phagemids were excised in vivo from the Lambda ZapII vector by the rapid excision procedure (Strategene, La Jolla, Calif.). Plasmid clones were subsequently transformed into the Sure strain of E. coli and restriction maps of the inserts were constructed. The restriction map of the 5.5 kb prf1-m3207 genomic clone is shown in FIG. 3, wherein the position of Sa-1, sequences representing a 254-bp Sac I-Alu I fragment, and SH-2, sequences representing a 2218-bp Sac I-Hin cII fragment, are illustrated below the map (E, Eco RI; X, Xho I; S, Sac I; a, Alu I; Hc, Hin cII; B, Bam HI; C; Cla I; D, Dra I; H, Hin dIII; Ac, Acc I; M, Mlu I; Bc, Bcl I. XX153 is an olignucleotide primer representing the Mu <u>terminal inverted repeat sequence</u>. KW487 and KW485 are primers that were used to amplify Sa-1. The low copy sequence, Sa-1, was amplified via <u>PCR</u> from Wf9-BG progenitor DNA with the KW487 and KW485 primers. The amplification product, which represents a 254-bp Sac I-Alu I fragment flanking the Mul insertion site in prf1-m3207, was used in DNA gel blot hybridization analyses to establish that the prf1-m3207 clone cosegregates with rfl locus. In contrast, although the 2.4-kb Mul-hybridizing Eco RI restriction fragment is linked to the rfl-m3310 allele, as indicated below, it does not appear to represent Rfl sequences.

DEPR:
Other significant portions of predicted amino acid similarity to the p6140-1 cDNA were to hypothetical protein 1 of the En/Spm <u>transposable</u> element system of <u>maize</u> (Rf1 6140-1 cDNA nucleotides 2-262; 73% homology, 47% identity) and En/Spm mosaic protein (Rf1 6140-1 cDNA nucleotides 316-582; 47% homology, 31% identity). Nucleotides 2-262 also had substantial similarity to other known <u>transposable</u> element systems from Glycine max (soybean) and Antirrhinum majus (garden snapdragon), though one skilled in the art recognizes that all of these <u>transposable</u> element systems are similar to one another.

DEPR:
The above results suggested that the Rfl mRNA may be chimeric, a result that would be consistent with predictions that Rfl represents a neomorphic allele (Wise et al. (1996), supra). Due to the diverse nature of the individual motifs within the Rfl p6140-1 cDNA, we predicted that this mRNA may originate via recombination between different domains from different genes. One test of this hypothesis was performed as follows. A DNA restriction fragment representing the region homologous to snRNP E (nucleotides 1355-1543) was isolated via double digestion of the p6140-1 cDNA with Mlu I and Bgl II. Sequences representing the plant structural-gene homologue (nucleotides 2077-2451) were amplified via polymerase chain reaction. Both of these fragments were used as individual hybridization probes on the original Wf9-BG cDNA library filters. Fourteen positive plaques (out of 400,000) hybridized to the snRNP E horiologue probe and more than 500 hybridized to the plant structural-gene homologue probe at high stringency. Except for hybridization to the original p6140-1 plaque, the two groups of the hybridizing plaques were mutually exclusive. All of the inserts identified via hybridization to the snRNP E homologue were of distinct size classes, whereas almost all of the inserts identified via hybridization to the plant structural-gene homologue were nearly identical. Additionally, the snRNP E probe hybridized to multiple restriction fragments on DNA gel blots of total <u>maize</u> DNA whereas the structural protein probe hybridized to low-copy sequences. These results suggested that the fourteen clones identified via hybridization to the snRNP E homologue may be discrete, yet related sequences, whereas the abundance of clones identified via hybridization to plant structural protein homologue may be identical but originate from one or two highly expressed sequences in etiolated seedlings, the tissue source of the Wf9-BG cDNA library. Eight plaques identified by each hybridization were purified, plasmids excised, and sequenced. Sequence alignment analyses suggest that the Rfl mRNA is a true chimera with one of the recombination breakpoints (between four cDNAs identified

via hybridization to the snRNP E homologue and eight cDNAs identified via hybridization to the plant structural-gene homologue) at nucleotide 1545.

DEPL:
In the absence of mutation, progeny kernels from each cross are heterozygous for the wild-type Rf allele and should, therefore, yield wild-type, i.e., male-fertile, plants. However, if a gamete from the transposon-carrying parent carries a <u>mutant</u> Rf allele generated by insertional mutagenesis of the transposon at (i.e., within or immediately adjacent to) the Rf locus, the progeny kernels are heterozygous for the mutant: Rf allele, designated rf-m (i.e., rf1-m for Rf1 and rf2-m for Rf2), and should, therefore, yield <u>mutant,</u> i.e., male-sterile, plants.

DEPL:
Seven heritable rf2-m alleles were derived from the above-described <u>Mutator</u> and Spm transposon stocks. The progenitor allele for each of the seven rf2-m alleles is as follows:

DEPV:
Cross 1: T Rf1/Rf1 rf2 wc1/rf2 wc1 (inbred R213).times.N rf1/rf1 Rf2 wc1/Rf2 wc1 <u>Mutator</u>

DEEQ:
T Rf1/Rf1 (<u>Mutator</u>).times.N rf1-B37/rf1-B37.

DETL:
_____ <u>Maize</u> populations used for mapping rf1 and rf2 with RFLP and visible markers Cross No. of Traits Population type progeny Parent 1 Parent 2 scored _____ Rf1 92 1267-68* BC.sub.1 96 R213-T/ g16-N Rf1- g16 [rf1rf1, mediated [Rf1rf1, Rf2Rf2] male fer- rf2Rf2].sup..diamond-solid. tility G16 92 1140-43 F.sub.2 102.sup..tangle-solidup. Q66-N g16-N g16 92 2117-118 [G16G16] [g16g16] RG1 92g 5029-63 TC 89 R213-T/ g16-N Rf1- (6 selected).sup..cndot. Acc731 [rf1rf1 mediated [Rf1rf1 rg1rg1, male fer- rg1Rg1, Rf2Rf2] tility Rg1 rf2Rf2] RF2A 91g 6222-30 BC.sub.1 41 R213-T rf2-m Rf2- [Rf1Rf1, 8904/ mediated rf2Rf2] R213-N male fer- [rf1rf1, tility rf2Rf2] RF2B 92 1101-05 BC.sub.1 903 (86 evalu- R213-T/ R213-N Rf1- and ated for RFLP wx-m8 ·[Rf1Rf1, Rf2- markers) [rf1Rf1, rf2rf2] mediated Rf2rf2] male fer- tility _____ *Pedigree numbers associated with this population .sup..diamond-solid. Parental genotype. See Wise et al. (1994), supra. .sup..tangle-solidup. Selected for homozygous g16. .sup..cndot. Ragged, malefertile plants, carrying a recombination between the rg1 and rf1 loci were selected.

DETL:
                                                    No.
of rf2-m Mutation rate alleles Population no./100,000 Population Subpopulation Transposon donor source* isolated size

gametes <u>Mutator</u> YA Mu.sup.4 outcross (1220) 1 8,500 P Mu.sup.2 outcross (1120) 1 12,000 G Mu.sup.1 outcrosses (1212, 1215, 1218, 1219) 3 5,000 OB Mu.sup.2 outcross (1118) 0 12,000 B Mu.sup.2 outcross (1121, 4938) 0 9,700 M Mu outcrosses (1207, 1216, 1222, 1224) 0 3,100 <u>Mutator</u> population total 5 50,300 9.9 Cy OA/BB 1230-1234, 3919-3921 1 28,000 Cy population total 1 28,000 3.6 Spm CV Revertants from c1-m5 0 20,000 c1-m5 "Control" 1 80,000 Spm population total 1 100,000 1.0

*Transposon donor sources are indicated by the pedigree numbers, Mu outcross, Mu.sup.2 outcross, and Mu.sup.4 outcross are defined by Robertson, Mol. Gen. Genet., 191, 86-90 (1983).

CLPR:
2. The nucleic acid of claim 1, wherein said plant is selected from the group consisting of <u>maize,</u> soybean, petunia, common bean, rapeseed, canola, cotton, safflower, peanut, palm, sorghum, rice, wheat, alfalfa, beet and sunflower.

CLPR:
3. The nucleic acid of claim 1, wherein said plant is selected from the group consisting of <u>maize,</u> rapeseed, rice, petunia, common bean, sunflower and sorghum.

CLPR:
7. The method of claim 5, wherein said plant is selected from the group consisting of maize, rapeseed, rice, and sorghum.

CLPV:
(a) generating a first plant, which is homozygous for a leaky mutant allele of Rf1, wherein Rf1 comprises SEQ ID NO:4, or a leaky mutant allele of Rf2, wherein Rf2 comprises SEQ ID NO:1 or 3;

CLPV:
(a) generating a first plant, which is homozygous for a leaky mutant allele of Rf1, wherein Rf1 comprises SEQ ID NO:4, or a leaky mutant allele of Rf2, wherein Rf1 comprises SEQ ID NO:1 or 3;

CLPV:
(f) backcrossing the male-sterile progeny of the male-sterile plant of (e) by a plant like the plant of (a) until a plant, which is homozygous for the leaky mutant allele of Rf1 or Rf2, is obtained.

ORPL:
Wright et al. Isolation and Characterization of male flower cDNA's from maize. The Plant Journal 3. (1) 41-49. MPSRCH analysis of NDA sequence, 1993.

ORPL:
Kamps, T., et al., Maize Genet. Coop. Newsl., 66, 45 (1992).